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RESEARCH ARTICLE

Comparative population genetics of two dominant plant species of high Andean wetlands reveals complex evolutionary histories and conservation perspectives in Chile's Norte Chico

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Abstract High Andean wetlands are naturally fragmented ecosystems that are impacted by anthropogenic activities. Although they constitute important reservoirs of biodiversity and ecosystem service providers, many aspects of their ecology are still unknown. In this study, we investigated the population genetic structure of two dominant and highly interactive plant species of high altitude wetlands, *Patosia clandestina* (Juncaceae) and *Carex gayana* (Cyperaceae), in 21 high Andean wetlands of Chile's Norte Chico. Using *rbcL* gene sequences and AFLP markers, we found that both species displayed low levels of within-wetland genetic diversity, high inter-population genetic differentiation, and spatially-dependent genetic variation arising from isolation-by-distance. The distance at which populations become genetically independent was of the same order of magnitude in both species (125–175 km). Despite these similarities, idiosyncratic spatial patterns were detected. *C. gayana* in the three most northeastern wetlands demonstrated marked differences relative to the rest of the populations, with the latter group following a latitudinal stepping-stone pattern. In *P. clandestina*, a genetic barrier was found to divide the northern and southern populations into two balanced groups, and spatial genetic variation was consistent with a hierarchical island model. The data indicate that each of the two species likely responded to different geological and ecological events, resulting in the definition of unique evolutionarily significant units in both. These results suggest that the implementation of global conservation programs at regional scales would likely result in the loss of important components of biodiversity in these ecosystems, and underscore the need for caution in designing effective conservation strategies.

Keywords AFLP · *Carex gayana* · Genetic diversity · *Patosia clandestina* · Population differentiation · Evolutionarily significant units

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Introduction

High Andean wetlands are naturally highly fragmented ecosystems located in springs and valley bottoms between 3000 and 4200 m above sea level along Andean hydrographic networks (Ruthsatz 2000). As a result of their geographic isolation, these ecosystems exhibit high levels of plant endemism (Squeo et al. 1994), and are particularly vulnerable to contemporary anthropogenic disturbances and climate change (Squeo et al. 2006).

Chile's Norte Chico (26–32°S) is part of the Chilean Winter Rainfall—Valdivian Forest biodiversity hotspot. High Andean wetlands of this region are important reservoirs of biodiversity and ecosystem services providers (Squeo et al. 2006). They play a critical role in the capture, storage and progressive release of water used for irrigation and human consumption (Rundel and Palma 2000; Deil 2005). These unique aquatic ecosystems are extremely fragile, and in northern Chile are undergoing rapid degradation

driven by changes in precipitation regimes and anthropogenic activities (briefly summarized in Squeo et al. 2006). This region of Chile is characterized by widespread mining activity along the Andes, which constitutes the most important anthropogenic threat to these ecosystems (Oyarzún and Oyarzún 2011), causing important local perturbations via mechanical destruction, water extraction, and/or water and soil contamination (Oyarzún et al. 2003; Pizarro et al. 2010; Parra et al. 2011; Alvial et al. 2012, 2013). Chile's Norte Chico is especially impacted by mining activities, with several large-scale projects in progress or under development (see examples in Parra et al. 2011; Alvial et al. 2012, 2013). This threat to high-altitude ecosystems in the region is of particular concern considering the density of mining concessions located within or near the high-altitude wetlands. For example, although most of the northern sites in this study are located within existing conservation areas, they are also within or very close (<3 km) to active mining sites (Table S1 in Supplementary Material). In addition, almost all the southern sites are located within mining concessions that may be exploited in the near future. This clearly shows that these ecosystems are at risk and require particular attention (Squeo et al. 2006). In general, one of the most common measures applied in restoration and conservation programs to compensate for habitat perturbation caused by mining activities is the translocation of specimens of the most affected plant species, requiring previous knowledge of their ecology to maximize translocation success (Batson et al. 2015). Nevertheless, despite the importance and fragile nature of high Andean wetlands, the immediate threats to their persistence, and the need to improve knowledge of their ecology to implement effective conservation programs, research on ecological patterns and processes in these ecosystems remains sparse (Junk 2013), being limited to vegetation characterization and two community studies in Chile's Norte Chico (Ruthsatz 1993; Squeo et al. 2006; Bertin et al. 2015, 2017). Particularly noteworthy is the lack of population genetic studies despite the high degree of fragmentation in these high-altitude ecosystems.

It is recognized that high-altitude plant populations have been influenced by historical climatic oscillations and soil movements, and had to adapt to the spatial isolation of suitable habitats (Kuss et al. 2008; Stöcklin et al. 2009; Thiel-Egenter et al. 2011). Reduced connectivity between populations in such fragmented habitats can result in little genetic exchange between populations, which may jeopardize population persistence and alter species interactions (Aguilar et al. 2006; Ibáñez et al. 2014). Indeed, when populations are restricted to small and isolated patches they are more prone to any kind of stochastic event (i.e. environmental, genetic and demographic). Small populations in particular tend to lose genetic diversity due to the effects of genetic

drift (Hedrick and Kalinowski 2000; Woodruff 2001), and increased inbreeding within patches often causes the reduction of individual fitness (Crandall et al. 2000; Jump and Peñuelas 2006; Ibáñez et al. 2014), which increases population extinction risk (Frankham et al. 2010). These population persistence factors assume still greater importance in the context of ecosystems impacted by anthropogenic pressures, as in the high Andean wetlands of Chile's Norte Chico threatened by mining activity. Thus, understanding the patterns of genetic variation within and between populations of key species in these highly fragmented ecosystems is necessary to support strategic decisions regarding the conservation and the management of these ecosystems' biodiversity, especially when the translocation of specimens between sites is required. Indeed, the analysis of spatial genetic structure can provide valuable information about the scale and magnitude of current and historical gene flow between populations and help identify populations with significant differences in allele frequencies, which is essential to delineate management units (MU) (Manel et al. 2003; Frankham et al. 2010).

In this study we investigate the genetic diversity and population structure of two dominant plant species of high Andean wetlands, *Carex gayana Desv.* (Cyperaceae) and *Patosia clandestina (Phil) Bucheneau* (Juncaceae), along the latitudinal range of Chile's Norte Chico (~600 km). Both are highly interactive species; *P. clandestina* is a vigorous peat-forming plant that hosts many other wetland plant species (Ruthsatz 1993; Squeo et al. 2006), while *C. gayana* constitutes a major grazing resource for wild mammals (Bonino and Borelli 2006). Because highly interactive species are ecologically pivotal at the community and/or ecosystem levels, they deserve special attention in conservation planning (Soulé et al. 2003). A comprehensive overview of population genetic patterns of *C. gayana* and *P. clandestina* will thus bring critical information for the long term conservation of high Andean biological diversity. Specifically, the aims of the present study were to (1) assess levels of genetic diversity in populations of these two plant species, (2) investigate the spatial pattern of their population genetic structure and (3) define possible MU for conservation strategies.

Materials and Methods

Study area and species

Chile's Norte Chico is located between 26 and 32°S (Fig. 1a). It includes five river basins: Copiapo, Huasco, Elqui, Limarí and Choapa from north to south. In this area high Andean wetlands are found in the low Alpine and sub-Alpine belts of the Central Andes at elevations above
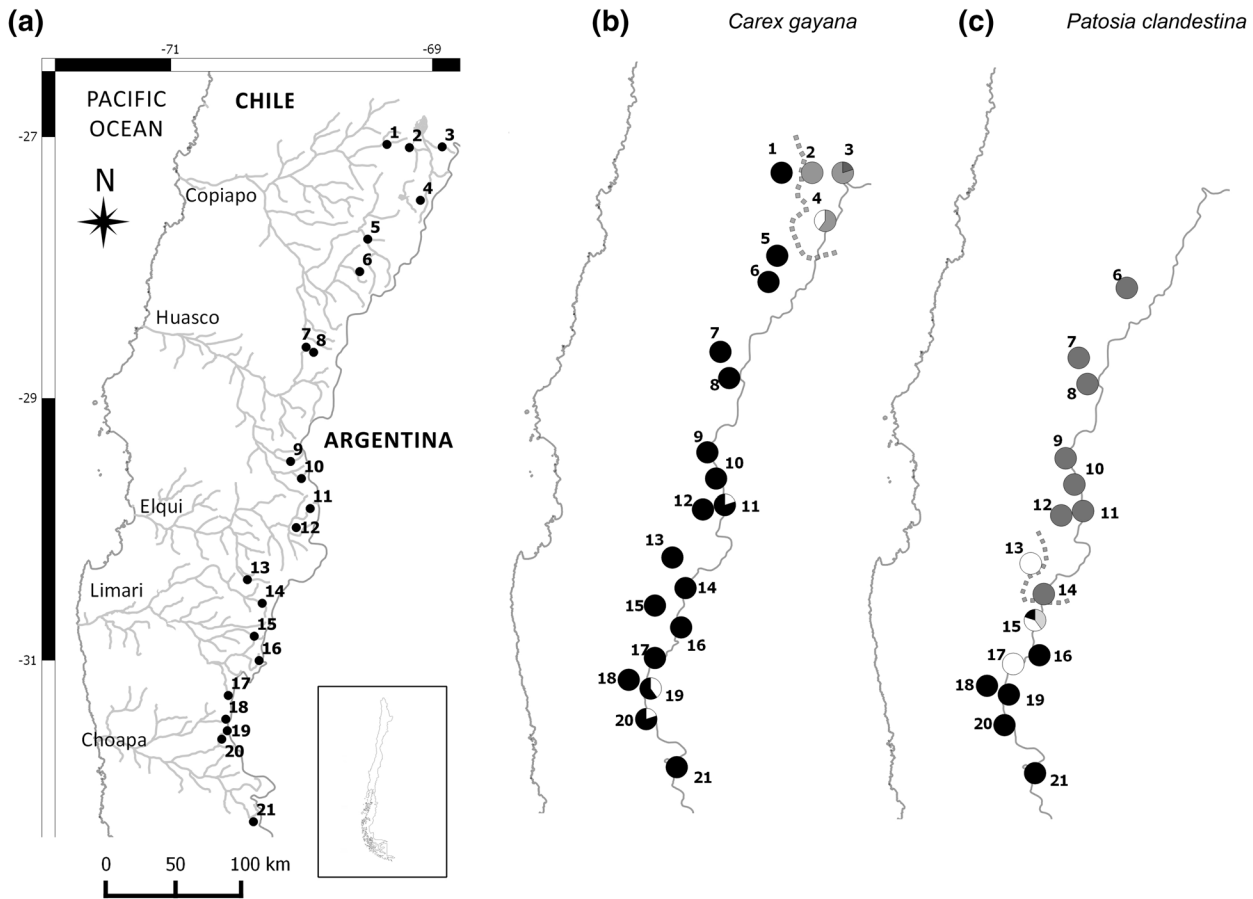
Fig. 1 Geographical location and *cpDNA* haplotype diversity of the high Andean wetlands sampled along Chile's Norte Chico. **a** Map of the study region showing the location of the 21 wetlands studied along the five main river basins in Chile's Norte Chico Andean Highlands; the country limits are represented in black lines and the main river basins are drawn in *grey lines*. Spatial distribution of the *rbcL* haplotypes of *Carex gayana* (**b**) and *Patosia clandestina* (**c**). Hap- lotypes are distinguished by *black, white, light grey* and *dark grey* colors (HC1, HC2, HC3 and HC4 for *C. gayana*), and (HP1, HP2, HP3 and HP4 for *P. clandestina*, respectively), and pie slices represent the relative frequency of each haplotype within each wetland. The main genetic boundaries between wetland populations inferred with Monmonier's maximum difference algorithm are depicted by a broken *bold line*

2800 m (Squeo et al. 2006). They face a wide range of topographic and environmental conditions, with a climate varying from arid in the north to Mediterranean in the south (Squeo et al. 2006). High Andean wetland vegetation has been described as hard cushion mires with temporary moorland pools (Deil 2005), and according to Ruthsatz (1993) *P. clandestina* (Juncaceae) and *C. gayana* (Cyperaceae) are dominant species in these ecosystems. Both species are described as perennial herbs that are widely distributed along the Andes of central and southern South America: *C. gayana* occurs throughout eastern South America in Peru, Bolivia, Chile and Argentina, including the high Andean wetlands of Chile's Norte Chico (Romand-Monnier 2013, The Global Biodiversity Information Facility: GBIF Backbone Taxonomy 2013); *Patosia clandestina* is restricted to the high Andes of Bolivia, northern and central Chile, and

from Jujuy to Neuquén (NW and SW) in Argentina (Wilson and Berendsohn 2017). With respect to growth form, *C. gayana* is rhizomatous (Kiesling 2009), whereas *P. clandestina* is highly caespitose, forming very hard, dense cushions (Barros 1953). Little is known about their breeding system; both *C. gayana* and *P. clandestina* display sexual reproduction and clonal propagation strategies (Bernard 1990 for the genus *Carex*, and van; Groenendael et al. 1996 and; Körner 1999 for Cyperaceae and Juncaceae), although they differ in certain aspects. *C. gayana* is monoecious (Barros et al. 1969), which favors a mixed breeding system that includes selfing and outcrossing; pollination in this species is mainly by wind, although the fact that male flowers are located at the apex of the stem and the female flowers at the base may allow for some degree of self-pollination (Gina Arancio, personal communication). By contrast, *P. clandestina* is

dioecious (Barros et al. 1969), and is therefore exclusively outcrossing. Pollination is most likely mediated by a combination of small insects and wind (Gina Arancio, personal communication).

Sample collection and DNA extraction

Sampling was carried out in 21 high Andean wetlands located along Chile's Norte Chico between March and April 2011 (Fig. 1a). We collected leaves from 30 individuals of *C. gayana* and *P. clandestina* in each wetland where they were present (i.e., 21 and 16 sites, respectively; Fig. 1; Table 1). Sampling was performed as evenly as possible in each site, considering a minimum distance of 3 m between plant samples. Plant samples were kept individually at room temperature in small plastic bags containing silica gel until DNA extraction. About 100 mg of dried plant tissue was ground with a Mini Beadbeater 16 (Biospec Products), and total genomic DNA was extracted following the CTAB protocol of Cota-Sánchez et al. (2006). DNA was resuspended in water, checked for integrity on 1% agarose gels stained with GelRed (Biotium), quantified with a NanoDrop 1000

spectrophotometer (Thermo Scientific) and then stored at −20 °C until PCR amplification.

cpDNA amplification and analysis

We amplified a 720 bp portion of the large chain of the chloroplast gene ribulose biphosphate carboxylase (*rbcL*) using the primers 1F and 724R (Fay et al. 1997). Five specimens of each species per site were amplified in 25 μl reactions containing 2.5 μl 10X buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 mM each primer, 1 μl BSA 1% w/v, 4% DMSO, 1 U Platinum *Taq* DNA polymerase (Invitrogen) and 100 ng genomic DNA. The cycling profile involved an initial denaturation at 94 °C for 5 min followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C, with a final extension of 5 min at 72 °C. Amplicons were sent to Macrogen Inc. (Seoul, Korea) for bidirectional sequencing.

The *cpDNA* sequences were edited manually and aligned using Geneious vR6 (Biomatters Ltd.). For each species, levels of *cpDNA* polymorphism were assessed with the DnaSPv5 program (Librado and Rozas 2009) using

Table 1 Population genetic diversity estimates in *Carex gayana* and *Patosia clandestina* populations along Chile's Norte Chico based on AFLP data

Basin	Wetland	*C. gayana*				*P. clandestina*			
		N	I	Hs	PLP	N	I	Hs	PLP
Copiapo	V1	29	0.15 ± 0.02	0.10 ± 0.02	32.69	–	–	–	–
	V2	30	0.04 ± 0.01	0.03 ± 0.01	14.42	–	–	–	–
	V3	29	0.08 ± 0.02	0.05 ± 0.01	23.08	–	–	–	–
	V4	29	0.13 ± 0.02	0.08 ± 0.02	27.88	–	–	–	–
	V5	30	0.10 ± 0.02	0.07 ± 0.01	25.00	–	–	–	–
	V6	29	0.24 ± 0.03	0.16 ± 0.02	58.65	29	0.14 ± 0.02	0.09 ± 0.02	43.21
Huasco	V7	30	0.21 ± 0.03	0.14 ± 0.02	46.15	30	0.17 ± 0.03	0.11 ± 0.02	48.15
	V8	29	0.13 ± 0.02	0.08 ± 0.02	28.85	29	0.14 ± 0.03	0.09 ± 0.02	33.33
	V9	29	0.13 ± 0.02	0.09 ± 0.02	28.85	17	0.12 ± 0.03	0.08 ± 0.02	30.86
	V10	30	0.14 ± 0.02	0.09 ± 0.02	44.23	27	0.19 ± 0.03	0.13 ± 0.02	51.85
Elqui	V11	30	0.14 ± 0.02	0.09 ± 0.02	33.65	16	0.14 ± 0.02	0.09 ± 0.02	37.04
	V12	30	0.14 ± 0.02	0.09 ± 0.02	31.73	28	0.13 ± 0.02	0.08 ± 0.02	40.74
	V13	19	0.16 ± 0.02	0.10 ± 0.02	36.54	28	0.19 ± 0.03	0.12 ± 0.02	44.44
Limarí	V14	30	0.14 ± 0.02	0.09 ± 0.02	32.69	28	0.17 ± 0.03	0.10 ± 0.02	50.62
	V15	30	0.11 ± 0.02	0.07 ± 0.02	27.88	29	0.22 ± 0.03	0.14 ± 0.02	53.09
	V16	30	0.09 ± 0.02	0.06 ± 0.01	20.19	29	0.17 ± 0.02	0.10 ± 0.02	54.32
	V17	29	0.15 ± 0.02	0.10 ± 0.02	31.73	30	0.13 ± 0.02	0.08 ± 0.02	49.38
Choapa	V18	30	0.22 ± 0.03	0.14 ± 0.02	51.92	27	0.17 ± 0.03	0.10 ± 0.02	48.15
	V19	15	0.41 ± 0.03	0.28 ± 0.02	67.31	29	0.20 ± 0.02	0.12 ± 0.02	66.67
	V20	30	0.24 ± 0.03	0.16 ± 0.02	48.08	24	0.12 ± 0.02	0.08 ± 0.02	32.10
	V21	22	0.18 ± 0.03	0.12 ± 0.02	36.54	29	0.16 ± 0.03	0.10 ± 0.02	44.44
Average values		28.05 ± 0.89	0.16 ± 0.01	0.10 ± 0.00	35.62 ± 2.83	26.81 ± 1.07	0.16 ± 0.01	0.10 ± 0.00	45.54 ± 2.35
Coefficient of variation		0.15	0.48	0.50	0.36	0.16	0.19	0.19	0.21

N Sample size, *I* Shannon's Information Index, *Hs* Nei's diversity index of expected heterozygosity, and *PLP* Polymorphic loci proportion

haplotype diversity (*Hd*) and nucleotide diversity (*Pi*) estimates. Nei genetic distances between sites were calculated with MEGA v6.06 (Tamura et al. 2013), and neighbor-joining (NJ) trees were performed in APE (Paradis et al. 2004) for both species. A rough approximation of the time of divergence between differentiated populations of *C. gayana* and *P. clandestina* was estimated with the algorithm of Nei and Kumar (2000) with a mutation rate (μ_{rbcL}) of 8.9×10^{-10} as described in Beatty and Provan (2012).

Note that we also tested additional chloroplast loci such as *matK* (Dunning and Savolainen 2010) and *trnH-psbA* (Starr et al. 2009), but their amplification turned out to be difficult with relatively poor sequence quality in both species. The few successful amplifications revealed no significant increase in haplotype diversity so these loci were not considered further.

AFLP protocol

AFLP amplifications were performed for all samples following the protocol of Meudt and Clarke (2007) available on the web at http://clarkeresearch.org/aflp_2012-01-26/aflp.html with very few modifications. We used 250 ng of genomic DNA for the restriction digest step, T4 DNA ligase from New England Biolabs for the linker ligation step, Platinum *Taq* DNA polymerase (Life Technologies) for the pre-amplification step and AmpliTaq DNA polymerase (Life Technologies) and 4 µl of pre-amplified DNA previously diluted 20X in water for the selective amplification step. In each species, four combinations of specific primers were selected based on their reliability and number of fragments amplified. The pairs AAC$_{6FAM}$/CA, ACC$_{NED}$/CT, AAC$_{VIC}$/CT were used in both *C. gayana* and *P. clandestina*, in addition to the pair ACA$_{PET}$/CT in *C. gayana* and ACA$_{PET}$/CA in *P. clandestina*. AFLP fragments were separated by capillary electrophoresis with a 3130 genetic analyzer (Life Technologies) and fragment size determined using the GeneScan 500-ROX size standard (Life Technologies). Genotyping was performed with the software GeneMarker v2.4 (Softgenetics). Prior to genotyping, all electropherograms were individually checked for calibration accuracy, and only fragments longer than 120 bp and smaller than 450 bp were considered for further analysis (Caballero et al. 2008). Bands were scored as present (1), absent (0), or "no data" (NA) when they could not be scored unambiguously. Reproducibility of each marker was checked by repeating the AFLP protocol for 10% of the samples. Because data quality can affect the reliability of population genetic parameter estimates (Herrmann et al. 2010), only markers that could be unambiguously scored for most samples were selected for genotyping. As recommended by Bonin et al. (2007), we retained only AFLP markers with a genotyping error rate lower than 10%,

calculated as the sum of "correct" (1,1 or 0,0) between two replicates divided by the total number of re-amplified individuals and multiplied by 100. Furthermore, loci were also tested for redundancy with the use of the AFLPOP 1.1 software (Duchesne and Bernatchez 2002). After filtering, only loci showing between 2 and 98% polymorphism were kept for further analyses.

AFLP data analysis

The find haplotypes option implemented in GeneAIEx v.6.5 (Peakall and Smouse 2012) was used to determine the presence of clonal individuals at each site. To estimate genetic diversity, we calculated the Shannon information index (*I*) (Lewontin 1972), Nei's genetic diversity index (*Hs*) (Nei 1973) and the proportion of polymorphic loci (*PLP*) using POPGENE 1.31 (Yeh and Boyle 1997). Genetic distances between populations were calculated using matrices of pairwise Φ_{ST} based on AFLP data with Arlequin v3.5.1.2 (Excoffier and Lischer 2010), and NJ trees were constructed using the package APE (Paradis et al. 2004) in the R environment (R Development Core Team 2014, http://www.R-project.com). Analysis of Molecular Variance (AMOVA) was performed using Arlequin v3.5.1.2 (Excoffier and Lischer 2010) to test the partitioning of genetic variance between river basins and between the northern and southern groups found in the NJ trees for both plant species.

Genetic structure was also assessed by performing Discriminant Analysis of Principal Coordinates (DAPC), an individual-based multivariate approach (Jombart et al. 2010). Since DAPC analysis requires prior definition of groups, we ran sequential *k-means* clustering with increasing values of *k* using the Bayesian Information Criterion (BIC) to determine the optimal number of clusters (*k*). Because no optimal *k* was found for our results, we used the number of populations instead. Prior to discriminant analysis, the data were reduced using principal component analysis, and the number of retained principal components (PCs) was chosen by optimizing the *a*-score (Jombart 2008). Finally, discriminant functions were constructed as linear combinations of the retained PCs. All these procedures were executed with the Adegenet v1.4-2 package (Jombart 2008) in R (R development core team 2014, http://www.R-project.com). To test the reliability of the DAPC results, we repeated the analysis with the Bayesian clustering method implemented in STRUCTURE v2.3.4 (Pritchard et al. 2000). We used an "admixture" model with a 50,000 burn-in period and 100,000 MCMC replicates after burn-in, with ten runs for each value of *k* (2 to 21 for *C. gayana* and 2 to 16 for *P. clandestina*). STRUCTURE outputs were then analyzed with STRUCTURE HARVESTER (Earl and von Holdt 2012), and we used Evanno's method to detect the most likely value of *k* (Evanno et al. 2005). The results

were highly concordant with those of DAPC, thus only the DAPC outputs are shown in the results.

We used Alleles in Space v1.0 (Miller 2005) to identify potential landscape genetic barriers, applying Monmonier's maximum difference algorithm (Manel et al. 2003). Isolation by distance (IBD), a measure of the dependence between geographic and genetic distances, was assessed by performing Mantel tests (Mantel 1967) and spatial autocorrelation analyses with Mantel correlograms in R. Correlation between latitude and genetic diversity indices was similarly assessed using R.

Effects of local characteristics on genetic diversity

We investigated the association between population genetic diversity and local characteristics including precipitation, wetland size and species population size. Precipitation was estimated as described in Bertin et al. (2015). Wetland size was inferred from GIS data (Bertin et al. 2017). As a proxy of size of the *C. gayana* and *P. clandestina* populations, we used species-specific relative biomass calculated from plant community samples collected from each wetland (Bertin et al. 2017). To examine the effects of local characteristics on genetic diversity, we conducted separate linear models on the distinct genetic diversity indices. Before running the linear models, the genetic diversity indices were log-transformed to comply with normality of residuals.

Results

rbcL Analysis

The *cpDNA* sequences of the *rbcL* gene portion for the individuals analyzed (104 and 79 for *C. gayana* and *P. clandestina*, respectively) comprised ca. 690 nucleotides for *C. gayana* and 659 nucleotides for *P. clandestina*. Four haplotypes were found in each species (Fig. 1b, c). Sequences were deposited in Genebank with the following accession numbers: KU746982, KU746983, KU746984, and KU746985 for *C. gayana*; KU746986, KU746987, KU746988, and KU746989 for *P. clandestina*.

The overall nucleotide diversity of *C. gayana* over the studied region was low (21 wetlands, $Hd = 0.32$, $Pi = 0.001$), and no deviation from neutrality was evident (Tajima's $D = -0.77$, $P > 0.10$). Only five of the 21 wetlands (V3, V4, V11, V19, and V20) showed polymorphism at the *rbcL* locus, with each harboring only two haplotypes (Fig. 1b). The most common haplotype (HC1) was present in 18 of the 21 wetlands (81.73% of the samples), whereas the other haplotypes were only sporadically detected. While HC2 was only observed in four wetlands, it was present over the whole study area. By contrast, the two remaining

haplotypes HC3 and HC4 were restricted to the three most northeastern wetlands (V2, V3 and V4) where HC1 was absent (Fig. 1b). Due to this singular haplotype composition, these three northeastern wetlands differed strongly from the rest of the sites (Fig. S1a), with a divergence time between the three northeastern wetlands and the rest of the sites estimated at 2.4 million years.

In *P. clandestina*, the four haplotypes differed only by three substitutions at positions 211, 352 and 643. Overall haplotype and nucleotide diversity were also low in this species ($Hd = 0.63$, $Pi = 0.001$), and no deviation from neutrality was detected (Tajima's $D = 0.65$, $P > 0.10$). Only one wetland (V15) located in the Limarí basin showed genetic variation at the *rcbL* locus and included three haplotypes ($Hd = 0.8$; $Pi = 0.002$). The haplotype distribution was more heterogeneous than that observed for *C. gayana*. The main haplotype (HP4), found in 50.6% of the samples, was only present in the northern part of the study area, up to the Elqui basin in the south (V6–V12, and V14). By contrast, the other three haplotypes (HP1, HP2 and HP3) were absent from the north and were only detected in the southern part of the study area (V13, and V15–V21) (Fig. 1c). As a result, a clear separation divided the eight northern and the eight southern wetlands into two groups (Fig. S1b), with a mean divergence time estimated at 1.7 million years.

AFLP Analysis

Mean error rate estimation of AFLP loci was 0.034 for *C. gayana* and 0.048 for *P. clandestina*. After filtering for polymorphic, reliable and non-redundant loci, 104 and 81 AFLP loci were used for the genetic diversity and structure analysis of *C. gayana* and *P. clandestina*, respectively. The number of genotyped individuals per population ranged from 15 to 30, with a total of 589 individuals for *C. gayana* and 429 for *P. clandestina* (Table 1). The search for clonal individuals revealed a total of 56 clones in *C. gayana*, with a mean of 2.66 clones per site, which corresponds to 9.5% of the total data set. In *P. clandestina*, only 11 clones were found, representing a mean of 0.69 clone per site and 2.5% of the complete data set. Because no noticeable difference was found in the genetic diversity estimates and the population differentiation analyses after removing the clones in each site (data not shown), we only report here the results obtained with the full data sets (i.e. including the clones) in each species.

Both species harbored relatively low levels of genetic variation, with mean Shannon and Nei diversity indices of 0.16 and 0.10, respectively (Table 1). While the proportion of polymorphic loci (*PLP*) tended to be higher in *P. clandestina* than in *C. gayana* (pairwise t-test: $t = -1.97$, $P = 0.07$) with mean values of 45.5 and 35.6%, respectively (Table 1), no differences in diversity were detected between

the two species for the Shannon Information and Nei diversity Indices (pairwise t-tests for Hs and for I: $t = 1.14$ and 0.86, $P = 0.27$ and 0.40, respectively). The diversity indices were more variable in *C. gayana* as indicated by coefficients of variation that were about twice those observed for *P. clandestina* (Table 1). While genetic diversity in *C. gayana* decreased significantly with decreasing latitude, this trend was not significant in *P. clandestina* (See correlation tests with latitude for *I, PLP* and *Hs* diversity indices in Table S2 of the Supplementary Material).

Levels of genetic differentiation between populations were high in both species. The mean Φ_{ST} values were greater than 0.5 over all populations (0.64 and 0.55, $P < 0.001$ for *C. gayana* and *P. clandestina*, respectively). Even wetlands fewer than 30 km apart showed relatively high levels of genetic differentiation with mean Φ_{ST} values reaching 0.30 and 0.19 for *C. gayana* and *P. clandestina*. Pairwise Φ_{ST} values ranged from 0.11 to 0.90 in *C. gayana*, and from 0.04 to 0.74 in *P. clandestina* (Table S3 and S4, respectively).

In agreement with the *rbcL* results, both the NJ tree and the DAPC in *C. gayana* showed a strong genetic differentiation between the three northeastern-most wetlands and the other sites (Fig. S1c, and Fig. 2a). This finding was further supported by the detection of a genetic barrier between these two clusters using Monmonier's maximum difference algorithm (Fig. 1b). According to the AMOVA analyses (Table 2), this subdivision explained 75% of the genetic variation over the whole area, whereas only 20% of the variation was accounted for by between basin effects. The DAPC scatterplot suggested that the southern group followed a stepping-stone structure (Fig. S2). This was corroborated by an overall positive association between population genetic and geographic distances for this group (Mantel correlation test: $r_M = 0.47$, $P = 0.001$,

Fig. S4a) and the shape of the Mantel correlogram, which demonstrated that genetic similarity between populations decreased continuously with increasing geographical distance (Fig. 3a). The Mantel correlations were positive and significant for the first three geographic distance classes, and negative and significant for the two most distant classes. The geographic distance at which genetic similarity ceased to be significant occurred for population pairs that were 125–175 km apart.

In *P. clandestina*, the results of the NJ tree were consistent with those of the *rbcL* analysis. Indeed, the NJ tree differentiated two groups comprised of the eight northern and eight southern wetlands, respectively (Fig. S1d). This genetic divergence was also largely supported by the genetic barrier detected through Monmonier's maximum difference algorithm (Fig. 1c) and the DAPC results (Fig. 2b). According to the AMOVA analysis, the divergence between the northern and southern clusters explained 46% of the total genetic variation, whereas only 22% of the variation was accounted for by between basin effects (Table 2). The second component of DAPC indicated, in addition, that the southernmost population (V21) was clearly differentiated from other populations within its own cluster (Fig. 2b). Overall, the shape of the DAPC scatterplot was consistent with a hierarchical island model with population clusters that strongly overlapped within both the northern and southern groups. A positive association between population genetic and geographic distances was observed (Mantel test, $r_M = 0.63$, $P = 0.001$, Fig. S4b). The correlogram displayed a stabilizing profile where significant genetic similarity was found among geographically close populations but rapidly became null (around 125 km, Fig. 3b). In fact, the Mantel correlation was negative and stabilized at values around -0.2 for population pairs more than 175 km apart.
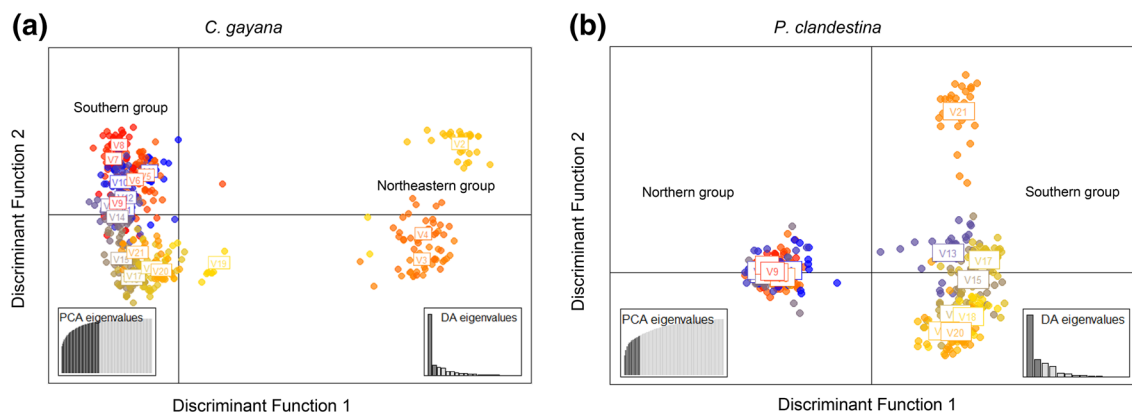

Fig. 2 Discriminant analysis of principal components (DAPC) based on AFLP data in *C. gayana* and *P. clandestina*. Graphs show **a** the divergence between the northeastern and southern groups, as well as the stepping-stone like variation within the southern group in *C. gayana*, and **b** the divergence between the northern and the southern wetlands in *P. clandestina*. (Color figure online)

Table 2 Analysis of molecular variance (AMOVA) based on hydrography and the identified DAPC groups in *Carex gayana* and *Patosia clandestina* along Chile's Norte Chico, using the AFLP data

Species	Grouping	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation (%)	Φ	P
C. gayana	By river basins	Among regions	4	1984	2.70	20	0.20	0.011
		Among populations within groups	16	2985	6.52	48	0.60	<0.001
		Within populations	568	2465	4.34	32	0.68	<0.001
	Northeastern vs. Southern groups	Among regions	1	3349	21.77	75	0.75	0.002
		Among populations within groups	19	1621	2.90	10	0.40	<0.001
		Within Populations	568	2465	4.34	15	0.85	<0.001
	By river basins within the southern group	Among regions	4	952	1.06	10	0.10	0.016
		Among populations within groups	13	1682	4.52	44	0.49	<0.001
		Within populations	483	2226	4.61	45	0.55	<0.001
P. clandestina	By river basins	Among regions	4	1226	2.27	22	0.22	0.013
		Among populations within groups	11	1246	4.13	40	0.51	<0.001
		Within populations	413	1630	3.95	38	0.62	<0.001
	Northern vs. Southern groups	Among regions	1	1327	5.81	46	0.45	<0.001
		Among populations within groups	14	1145	2.91	23	0.42	<0.001
		Within populations	413	1631	3.95	31	0.69	<0.001
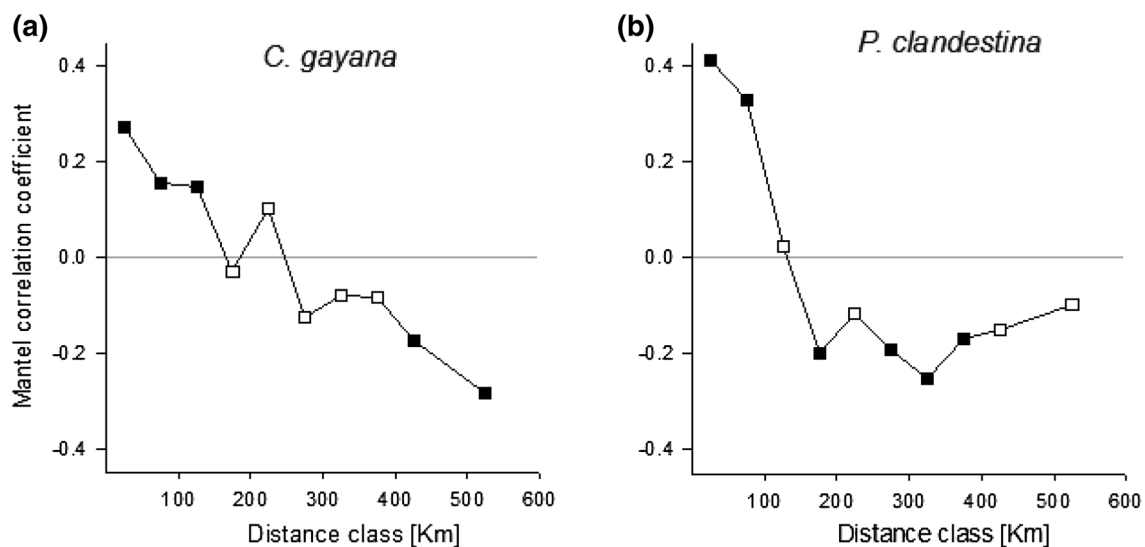
Fig. 3 Mantel correlograms based on AFLP pairwise Φ_{ST} estimates in *C. gayana* (**a**) and *P. clandestina* (**b**). Black squares correspond to statistically significant Mantel correlation coefficients

Effects of local characteristics on genetic diversity

Linear models revealed little evidence of effects of local characteristics on plant genetic diversity. For *P. clandestina*, none of the models were statistically significant [adjusted R^2 range: (0.05, 0.20), $F_{3,12}$ range: (1.3, 1.8),

$P > 0.05$ for all three genetic diversity indices]. In *C. gayana*, the linear model conducted on the Shannon diversity index was significant (adjusted $R^2 = 0.26$, $F_{3,12} = 3.34$, $P = 0.04$), revealing a negative effect of biomass on the genetic diversity estimate (Coefficient estimate $\pm$ standard error: $-3.6 \times 10e{-}03 \pm 1.5 \times 10e{-}03$, $t = -2.34$, P = 0.03).

Although only borderline significant, the same trends were observed regarding *Nei*'s genetic index and the proportion of polymorphic loci [*t* range: (−2.1, −1.96), P range: (0.05, 0.06)].

Discussion

Our study reports the first population genetic analysis of high Andean wetland plant species. It shows common genetic patterns between the plant species *C. gayana* and *P. clandestina*, since both displayed low levels of genetic diversity within wetlands associated with high inter-population genetic differentiation. However, our study also revealed idiosyncratic features regarding the spatial pattern and structure of genetic variation in these species.

Factors affecting within-population genetic diversity

The low levels of genetic diversity observed in both species are concordant with those described in other species inhabiting high-altitude ecosystems (e.g. Wang et al. 2015; Dantas et al. 2015; Körner 1999). In such ecosystems, plant breeding system, demographic history and reduced gene flow have been recognized as the main factors responsible for low levels of genetic diversity (Meloni et al. 2013; Körner 1999; Wang et al. 2015). In high altitudes, plants have developed mixed reproductive strategies (sexual and asexual reproduction) that cope with the high environmental variability (Körner 1999). Although self-fertilization as well as vegetative propagation can severely affect levels of genetic diversity in plant species (Hamrick and Godt 1996), negligible effects can be expected as long as they occur simultaneously with sexual reproduction and the rate of clonal propagation and/or selfing is low (Balloux et al. 2003). Both *C. gayana* and *P. clandestina* possess a combination of sexual reproduction and clonal propagation strategies (Bernard 1990 for the genus *Carex*, and van; Groenendael et al. 1996; Körner 1999 for Cyperaceae and Juncaceae). Nevertheless, the presence of clonal individuals was relatively low in our study (9.5 and 2.5% for *C. gayana* and *P. clandestina*, respectively) and no difference was found between the AFLP genetic diversity indices calculated with and without clones (data not shown). Thus vegetative propagation is unlikely to explain by itself the low levels of genetic diversity observed in these species. However, although no differences in the genetic diversity indices between the two species could be distinguished, they differed slightly in their proportion of polymorphic loci (45.5 vs. 35.6% in *P. clandestina* and *C. gayana*, respectively), which may reflect some differences in their respective breeding systems and growth habits. Indeed, the levels found in each species are consistent with those revealed by Reisch and Bernhardt-Römermann (2014) for plant species with mixed (i.e. outcrossing and selfing) or outcrossing mating systems. As the proportion of polymorphic markers is expected to be higher in outcrossing species than in species with a mixed mating system, our results provide evidence for an outcrossing strategy in *P. clandestina* and a mixed mating strategy in *C. gayana*, in line with other data from the literature. The higher proportion of clones occurring in *C. gayana* relative to *P. clandestina* is another argument in favor of a mixed mating strategy in this species, since it is likely a result of its rhizomatous growth. Regarding the influence of growth habit on population genetic diversity, it has been found that populations of caespitose species such as *P. clandestina* typically harbor lower levels of genetic diversity relative to those of rhizomatous species, such as *C. gayana* (Bruederle et al. 2008); this is not the case in our study however, with both species exhibiting comparable genetic diversity levels. It is also important to note that the very low levels of genetic diversity revealed by the genetic diversity indices and the relatively high levels of population differentiation are similar to those seen in selfing species (Nybom 2004; Reisch and Bernhardt-Römermann 2014), although our species clearly are not predominantly selfing and rather suggests that other factors, more specific to high altitude species, are likely to be responsible for such patterns.

Both historical and more recent demographical events may have contributed to modern-day genetic diversity in *C. gayana* and *P. clandestina* populations. Indeed, loss of genetic diversity due to drastic population bottlenecks during the Quaternary glaciations is well documented (Avise 2000; Hewitt 2000). There is an extensive history of glaciations along the Andes, including Chile's Norte Chico (Messerli et al. 1993). Although this region of Chile was not covered by the ice shield during the main glaciation periods, periodic glaciation events occurred over the area since the Pliocene (5.1–2 MYA, Clapperton 1979) and throughout the Pleistocene (2 MY–10,000 years BP) (Messerli et al. 1993). According to paleoclimatic and paleoecological evidence, high Andean wetlands from Chile's Norte Chico suffered local surface contractions during glacial expansion (Maldonado et al. 2005; Maldonado and Rozas 2008; Zech et al. 2007). This is also corroborated by mapping the wetland sites over the climatic layer of the Last Glacial Maximum period (ca. 22,000 years ago) obtained from the Worldclim—Global Climate Data v.1.4 (http://www.worldclim.org, Hijmans et al. 2005) (Fig. S3), which suggests that some of these high-altitude wetlands could have been completely covered by ice. Thus it is likely that strong population bottlenecks caused by local surface retraction in the relict wetlands have led to loss of genetic variation in

high Andean wetlands populations, as extensively reported in high altitude plant populations (Wang et al. 2015).

More recent demographic events may have accentuated the loss of population genetic variation in both species as well. Indeed, small isolated populations are more prone to lose genetic diversity due to genetic drift and inbreeding (Woodruff 2001; Hedrick and Kalinowski 2000; Wang et al. 2015). High-Andean wetlands are naturally fragmented ecosystems scattered within the Andes (Squeo et al. 2006). Thus, their biological populations are likely to remain relatively isolated. The Φ_{st} values in our study were high overall even between populations that are relatively close geographically. Populations with limited gene flow are particularly vulnerable to the effects of demographic stochasticity. In Chile's Norte Chico, sharp variations in plant demography occur periodically due to the strong influence of the El-Niño Southern Oscillation (ENSO), which generates major fluctuations in precipitation regimes (Rutlant and Fuenzalida 1991) and hence in plant productivity. Drastic reductions in primary productivity in these ecosystems during the dry years following each El-Niño event (Squeo et al. 2006) may indeed have a strong impact on high Andean wetland plant populations, leading to loss of genetic variation. There is unfortunately no data on recent trends in wetland size reduction due to anthropogenic or climate changes, although a recent study confirms a decline in precipitation in this region of Chile, and predicts greater persistence and recurrence of future drought events (Boisier et al. 2015). Under such a scenario, major declines in the extent and accelerated deterioration of these fragile ecosystems would be anticipated (Squeo et al. 2006).

While both species displayed low genetic diversity levels within wetlands, only *C. gayana* showed a strong decrease in genetic diversity from south to north. Given that Chile's Norte Chico was not covered by the ice shield during the main glaciation periods, and that latitudinal range contractions occurred northward in southern South American species during the Quaternary glaciations (Turchetto-Zolet et al. 2013), implying that northern populations usually harbor greater genetic diversity than southern populations, the pattern found in *C. gayana* cannot be explained by the recolonization process from a southern refuge during this period. The density of high altitude wetlands decreased from north to south (Bertin et al. 2017) following a decreasing precipitation gradient. Because *C. gayana* is not purely aquatic, in contrast to *P. clandestina*, which grows and remains in permanent watercourses, we could expect the presence and abundance of *C. gayana* to depend more strongly on precipitation levels. This suggests that population size of *C. gayana* would decrease along these latitudinal gradients, and that smaller northern populations would be more prone to genetic drift than those from the

south (Woodruff 2001). Contrary to these expectations, we did not find evidence for any positive relationship with precipitation nor with a possible proxy of population size (e.g. wetland size and *C. gayana* biomass) on this species' genetic diversity. In fact, our analyses revealed a significant negative relationship between *C. gayana´s* relative biomass in the wetland plant community and genetic diversity. Overall, these results indicate that the latitudinal gradient in precipitation is unlikely to explain the observed decrease in genetic diversity from south to north in this species. Alternatively, the gradient in wetland isolation could explain this pattern. In a recent study, Bertin et al. (2017) identified wetland connectivity as a major determinant of within-species genetic diversity in high Andean wetlands, with diversity being influenced positively by the number of surrounding wetlands but negatively by their mean proximity. The detected effects were particularly strong in *C. gayana* (i.e. explaining about 50% of the variance in genetic diversity between wetlands), suggesting that this species is highly sensitive to wetland isolation. By contrast, their influence was more moderate in *P. clandestina*, for which they only explained 19% of the variance in neutral genetic diversity.

Population genetic differentiation and structure across Chile's Norte Chico

Life history traits associated with both dispersal capacities and historical events may strongly affect the population genetic structure of high-alpine plant species (Meirmans et al. 2011). In our study, both *C. gayana* and *P. clandestina* exhibited high population genetic differentiation along Chile's Norte Chico and showed a barrier of deep genetic divergence between geographically close populations. In *P. clandestina*, the genetic barrier had a central location, dividing the northern and southern populations at the level of the Limarí basin. This barrier coincides in latitude with both the phylogeographic break uncovered in a red alga distributed along the coasts of Chile (Montecinos et al. 2012) and a strong divergence observed in the composition of benthic macroinvertebrate assemblages of high Andean wetlands (Bertin et al. 2015). This geographic area is recognized as a biogeographic transition zone in Chile for both terrestrial and marine biotas (Squeo et al. 2001; Hechem et al. 2011; Montecinos et al. 2012; Haye et al. 2014). The timing of the divergence was roughly estimated to be about 2 MYA, which indicates that it could be related to ancient geological and/or climate changes. Although such estimations must be taken cautiously, especially with a slow-evolving gene like *rbcL*, it is interesting to note that it falls within the range of divergence time estimated for the phylogeographic break reported in the red alga (Montecinos et al. 2012).

In *C. gayana*, the zone of genetic divergence was found in the north of Chile's Norte Chico, separating the three northernmost high altitude wetlands from the rest. The northern cluster belongs to the Laguna Santa Rosa and Laguna del Negro Francisco endorheic complex (Nevado Tres Cruces National Park, 27°17′S–69°08′W), which is composed of two brackish water lagoons united by the Pantanillo-Ciénaga Redonda biological corridor (all part of RAMSAR site 877, https://rsis.ramsar.org/ris/877). This hydrological complex, located at the southern end of the Chilean Altiplano, is completely independent from the Copiapó basin and has probably been isolated from it for several million years. Indeed, the last geological movements in the southern part of the Altiplano have been estimated at about 2 MYA (Elger et al. 2005), which would coincide with our time divergence approximation from the *rbcL* sequence data.

In addition to revealing genetic barriers, we found significant relationships between geographic and genetic distance in *C. gayana* and *P. clandestina*. In both cases the scatterplots of the genetic versus geographic distances indicate that the spatial dependence of genetic variation arose from IBD rather than from the presence of barriers to gene flow (Guillot et al. 2009) (Fig. S4). This result suggests genetic connectivity between geographically close populations. The distance at which populations become genetically independent was of the same order of magnitude for both species (i.e. 125–175 km), which may result from common dispersal mechanisms. Wind is one of the main large-scale dispersal mechanism (Van der Pijl 1982), and its importance for plant dispersal, by means of pollen flow in particular, has been demonstrated (i.e. Liepelt et al. 2002; Millar et al. 2014). Recently, Bertin et al. (2015) provided evidence for the role of wind as a dispersal factor of macroinvertebrate species in these high Andean ecosystems. Whether direct or indirect (i.e. by phoresy), wind-mediated dispersal of pollen may maintain some degree of gene flow between adjacent populations of both species, which is highly probable since these high-altitude ecosystems are characterized by very strong wind flows (Squeo et al. 2006).

Despite the presence of IBD structure in both *C. gayana* and P. *clandestina*, the patterns of genetic variation fit different population genetic models. Indeed, while the DAPC scatterplot in *C. gayana* was consistent with a stepping stone model, it suggested a hierarchical island model in *P. clandestina*, which indicates that population connectivity depends not only on geographic distance in this species but also on local population size. The idiosyncratic patterns of genetic differentiation revealed in *C. gayana* and *P. clandestina* show that these species had different evolutionary histories, likely responding to different geological and ecological events. These results bring new evidence that this

region of Chile has been the site of important and complex evolutionary events, which can explain its high levels of biodiversity and endemism (Squeo et al. 2001).

Conservation relevance

High-altitude wetlands of Chile's Norte Chico are vulnerable to anthropogenic threats such as mining activities and climate change (Junk 2013). From a conservation perspective, this study introduces new insights critical to conservation/restoration best practice in these ecosystems, in which compensatory approaches that involve translocation of individuals from the most vulnerable plant species are typically adopted. Indeed, the deep genetic divergence between northern and southern populations in each species, shown by both nuclear and chloroplast genetic markers, indicates that each group belongs to an independent evolutionary lineage and can be considered as a distinct evolutionarily significant unit (ESU, Moritz 1994). The fact that *C. gayana* and *P. clandestina* harbor different patterns of genetic differentiation along this region also reveals that implementing global conservation programs at a regional scale in these ecosystems is no easy task and the application of 'one size fits all' strategies could lead to the loss of important components of biodiversity. According to the Mantel correlograms, the minimum distance needed to conserve genetic diversity with maximum efficiency was estimated at 125–175 km in both species. For *C. gayana*, a long-distance cline was observed, indicating that genetic similarity steadily decreased with geographic distance. Thus to conserve most of *C. gayana*'s genetic variability in the overall area, about five MU separated by 125 km each should be considered (Diniz-Filho and De Campos Telles 2002), which could include the three northernmost wetlands as one distinct ESU and four MUs within the other ESU composed of the remaining wetlands. By contrast, genetic dissimilarity in *P. clandestina* stabilized for geographic distances greater than 175 km. In this case, considering the most distant populations to define MU would not allow preserving most genetic diversity (Diniz-Filho and De Campos Telles 2002). The selection of MU within each ESU instead requires identifying populations presenting genetic variability of particular interest.

Our work also provides relevant information for environmental impact assessment and biodiversity offsetting of high altitude wetlands. Under the existing legal framework (Supreme Decree No. 40, 2012 of the Environment Ministry), environmental licenses provided by the Chilean Environmental Impact Agency are required before developers can implement activities, and a mitigation hierarchy should be applied. However, the current legislation does not provide adequate protection of wetlands (Möller and Muñoz-Pedreros 2014), as existing decrees only apply to some components of

these ecosystems (wild species, water quality and quantity), but not wetlands as a whole. According to current legal standards (e.g. protection and restriction values of legal standards), high Andean wetlands rank among the least protected inland wetland systems in the country (Möller and Muñoz-Pedreros 2014). This further stresses the necessity to establish specific guidelines for environmental impact assessment and mitigation of these ecosystems in particular.

Neutral genetic variation is a biodiversity component of interest in conservation planning. It actually reflects population demographic characteristics, which is linked to risks of inbreeding and genetic drift. Both processes in turn reduce population viability (Frankham et al. 2010). The importance of high intrapopulation genetic diversity of *C. gayana* and *P. clandestina* for the conservation of high Andean wetlands is further underscored by the existence of strong and positive correlations of these genetic components with intra-wetland plant species richness (i.e. Pearson coefficient correlations greater than 0.6; Bertin et al. 2017). Plant diversity may be linked to a wide range of ecosystem services (Isbell et al. 2011) and may actually be linked to ecological functions in high Andean wetlands of Chile's Norte Chico (Roque 2015). Biodiversity management of high Andean wetlands should thus consider conserving habitat and landscape characteristics that are beneficial to plant genetic diversity. Neither wetland size nor precipitation was found to influence genetic diversity in the two plant species, suggesting that these characteristics might not be of prime importance. Bertin et al. (2017) identified wetland connectivity as a strong determinant of species and genetic diversity in high Andean wetland including for *C. gayana* and *P. clandestina* genetic diversity. To our knowledge, wetland connectivity / isolation is typically ignored in environmental impact assessments and planning for establishing adequate compensatory habitat strategies. We suggest that it should be considered as an ecological equivalence requirement to mitigate and repair biodiversity losses caused by the mining sector and grazing practices in high Andean wetlands.

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